

AD\_\_\_\_\_

Award Number: W81XWH-05-1-0051

TITLE: Anti-Angiogenic Action of Neutral Endopeptidase

PRINCIPAL INVESTIGATOR: David M. Nanus, M.D.

CONTRACTING ORGANIZATION: Weill Medical College of Cornell University  
New York, NY 10021-4870

REPORT DATE: November 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. <b>PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.</b>					
1. REPORT DATE (DD-MM-YYYY) 01-11-2005		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 1 Nov 2004 – 31 Oct 2005	
4. TITLE AND SUBTITLE Anti-Angiogenic Action of Neutral Endopeptidase				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-05-1-0051	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) David M. Nanus, M.D.  E-mail:				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Weill Medical College of Cornell University New York, NY 10021-4870				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Abstract follows.					
15. SUBJECT TERMS Angiogenesis, Cell surface peptidase, Neutral endopeptidase, Basic fibroblast growth factor, Prostate cancer Proteolysis					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT  UU	18. NUMBER OF PAGES  19	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

### **Abstract**

Angiogenesis, or the formation of new blood vessels from existing vasculature is an important event in tumor progression. It results from a complex, multistep biochemical cascade that is initiated by the activation of endothelial cells in response to angiogenic factors. In prostate cancers, angiogenic factors are produced by epithelial and stromal cells, and are believed critical to prostate cancer growth and progression. One of the most important of these factors is basic fibroblast growth factor (bFGF), which plays an important role in angiogenesis through the stimulation of endothelial cell proliferation, migration, and protease production (this is more of an *in vitro* phenomenon). A number of studies both *in vitro* and in patient specimens suggest that enhanced expression of bFGF contributes to more aggressive prostate cancer. Clearly, a better understanding of the pathways regulating angiogenesis in the prostate and how these pathways change during malignant transformation and prostate cancer progression will assist in developing more effective therapies for patients with prostate cancer.

Cell-surface peptidases are the guardians of the cell against small stimulatory peptides, functioning to control growth and differentiation in normal cells by regulating peptide access to their cell-surface receptors. They are integral membrane proteins with their enzymatic site exposed to the external cell surface. Neutral endopeptidase (NEP) is a cell-surface peptidase normally expressed by prostatic epithelial cells, whose expression is lost in over half of prostate cancers. NEP substrates include small peptides that have been implicated in prostate cancer progression, including endothelin-1, bombesin and neurotensin. We have recently discovered that bFGF is also a substrate for NEP. This is unexpected as previous NEP substrates have been considerably smaller in size (<35 amino acids compared with >150 amino acids for bFGF).

The goals of this application are to decipher the interaction between NEP and bFGF. Specifically, we proposed to understand how NEP cleaves bFGF and the resultant effects on angiogenesis of bFGF cleavage by NEP. We have now identified precisely how and where NEP cleaves bFGF, and the biological effects of these cleavage products on prostate cancer cells and human vascular endothelial cells. We are in the process of comparing angiogenesis in mice that have been engineered to lack the NEP gene allowing us to directly determine the contribution of NEP to angiogenesis. Studies are ongoing to assess the role of hypoxia on NEP action, and establish the relationship between NEP and other angiogenic factors such as heparin sulfate proteoglycans, vascular endothelial growth factor, and endothelin-1.

The results generated by our research are likely to contribute to a greater understanding of prostate cancer angiogenesis, and to explain, at least in part, the impact of NEP loss on bFGF expression. Moreover, this understanding potentially will have widespread applicability to not only other cancers, but to other angiogenic processes such as wound healing and ischemic vascular disease, where augmentation of angiogenesis (through inhibition of NEP) would be of therapeutic benefit. This improved understanding of NEP, bFGF and angiogenesis in prostate cancer may then be used in the design of novel therapeutic approaches.

## Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	5
Body.....	6
Key Research Accomplishments.....	9
Reportable Outcomes.....	9
Conclusions.....	10
References.....	11
Appendices.....	13

## Introduction

Neutral endopeptidase 24.11 (NEP, CD10, neprilysin) is a 90-110 kD cell surface peptidase normally expressed by a variety of tissues, including epithelial cells of the prostate, kidney, intestine, endometrium, adrenal glands and lung. This enzyme cleaves peptide bonds on the amino side of hydrophobic amino acids and inactivates a variety of physiologically active peptides, including atrial natriuretic factor, substance P, bradykinin, oxytocin, Leu- and Met-enkephalins, neurotensin, bombesin, endothelin-1 (ET-1), and beta amyloid. Loss or a decrease in NEP expression have been reported in a variety of malignancies, including renal cancer, invasive bladder cancer, poorly differentiated stomach cancer, small cell and non-small cell lung cancer, endometrial cancer and prostate cancer (Osman et al., 2004; Papandreou et al., 1998). Reduced expression of cell-surface peptidases such as NEP results in the accumulation of higher peptide concentrations that mediate neoplastic progression (Nanus, 2003).

Using prostate cancer as model to study the involvement of NEP in malignancy, we have demonstrated the following: 1) NEP protein expression is absent in nearly 50% of primary PCs (Osman et al., 2004); 2) NEP inhibits neuropeptide mediated cell growth, cell migration, and ligand-independent activation of the insulin-like growth factor-1 receptor (IGF-1R) leading to Akt phosphorylation (Papandreou et al., 1998; Sumitomo et al., 2001); 3) NEP can inhibit cell migration independent of its catalytic activity via protein-protein interaction of NEP's cytoplasmic domain with tyrosine-phosphorylated Lyn kinase, which then binds the p85 subunit of phosphatidylinositol 3-kinase (PI3-K) resulting in an NEP-Lyn-PI3-K protein complex. This complex competitively blocks the interaction between focal adhesion kinase (FAK) and PI3-K (Sumitomo et al., 2000); 4) NEP directly binds to ezrin/radixin/moesin (ERM) proteins resulting in decreased binding of ERM proteins to the hyaluronan receptor CD44 such that cells expressing NEP demonstrate decreased cell adhesion and cell migration (Iwase et al., 2004); 5) NEP directly interacts with the PTEN tumor suppressor protein, recruiting endogenous PTEN to the cell membrane, leading to prolonged PTEN protein stability and increased PTEN phosphatase activity, and resulting in a constitutive down regulation of Akt activity (Sumitomo et al., 2004); and 6) NEP expression inhibits tumorigenicity in an animal model of PC (Dai et al., 2001). Taken together, these studies have demonstrated that NEP protein functions to suppress and inhibit many processes that contribute to neoplastic progression.

In addition to epithelial cells, enzymatically active NEP is also expressed by vascular endothelial cells of venous and arterial origin (Llorens-Cortes et al., 1992). The NEP substrate ET-1 has previously been shown to act directly on endothelial cells via the ET<sub>B</sub> receptor to modulate different stages of neovascularization, including proliferation, migration, invasion, protease production and morphogenesis, resulting in neovascularization *in vivo* (Bagnato and Spinella, 2003). Based on these observations, we investigated whether NEP also functions as an antagonist of angiogenesis. We report that NEP is indeed antiangiogenic *in vivo*, significantly inhibiting angiogenesis. Surprisingly, we demonstrate that NEP catalytically inactivates the potent angiogenic factor basic fibroblast growth factor (bFGF; FGF-2), and that this is antagonized by heparin sulfate, consistent with a modulatory role for heparan sulfate proteoglycans/ This is the first report of an enzyme that cleaves bFGF *in vivo*, further demonstrating the potent tumor suppressive actions of NEP. NEP expression in PC cells is negatively regulated by hypoxia, consistent with its antiangiogenic function.

## Body

### a. Specific aims

The objective of this research is to continue to elucidate the mechanisms by which neutral endopeptidase 24.11 (NEP) functions to inhibit angiogenesis. These proposed studies should provide significant new knowledge on the multifunctional role of NEP as an inhibitor of bFGF-induced angiogenesis as well as clarify the role of the N-terminal cytoplasmic domain with regard to its regulation of angiogenesis. The aims have not been modified. The specific aims are:

- (1) To characterize the interaction between NEP and bFGF.
- (2) To delineate the anti-angiogenic action of NEP on human vascular endothelial cells.

### b. Studies and Results

During the initial year of grant support, we have commenced studies analyzing the antiangiogenic role of NEP on vascular endothelial cells. We have for the first time shown that the basic fibroblast growth factor is a substrate for NEP, and demonstrated mechanistically that proteolytic cleavage of bFGF by NEP abrogates signaling by producing inactive cleavage products.

*AIM I: Characterization of the interaction between NEP and bFGF.*

**Identification of a NEP cleavage site on bFGF.** To test the hypothesis that NEP could regulate angiogenesis in vivo, we used the murine corneal pocket assay to study the effect of recombinant NEP (rNEP) on bFGF-induced neovascularization. In this assay, pellets containing various concentrations of bFGF were implanted in the cornea ~1 mm from the limbus and neovascularization measured five days later. As shown in Figure 1, rNEP significantly inhibited bFGF-induced neovascularization ( $P < 0.01$ ). These results suggested the possibility that bFGF was inactivated by NEP via catalytic processing of the bFGF protein. Basic FGF is a potent proangiogenic, heparin-binding growth factor, with a primary translation product of 155 amino acids. NEP hydrolyzes peptide bonds on the amino side of neutral residues, however, a protein of 155 amino acids is theoretically too large to be a substrate for NEP as previous identified substrates are less than 43 amino acids (Howell et al., 1995; Kenny, 1993). To test whether NEP could hydrolyze bFGF, we incubated rNEP with recombinant bFGF for one hour and separated the products on a 14% polyacrylamide gel. Recombinant vascular endothelial growth factor (VEGF) was used as control. As shown in Figure 2A (lane 1, arrow), the molecular weight of bFGF protein but not VEGF protein was appreciably lower following rNEP incubation. The increased electrophoretic mobility of bFGF incubated with rNEP was blocked by the specific NEP inhibitor CGS24592 (Maniara et al., 1998), indicating that NEP and not a contaminating protease cleaved bFGF (Figure 2B, lane 4).

To confirm that NEP cleaves bFGF, we incubated bFGF and rNEP with and without the NEP inhibitor CGS24592, and analyzed the reactions using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. This identified a specific 2019 Da band that corresponded to a 20 amino acid peptide located at the C-terminus of the bFGF protein (Figure 2C). As a control, we performed the same digestion using either immunoprecipitated wildtype NEP (WT5) or enzymatically inactive NEP (M22) expressed using tetracycline-repressible promoter (Sumitomo et al., 2001) demonstrating that an intact enzyme activity is required to observe bFGF cleavage (Figure 2D). Analysis of the bFGF amino acid sequence

confirmed a potential NEP cleavage site between leucine 135 and glycine 136 (Abraham et al., 1986). Moreover, analysis of the amino acid sequence and the 3-dimensional structure of bFGF (Kastrup, 1997) indicated that the NEP recognition site between leucine 135 and glycine 136 was located at the outer edge of the bFGF protein suggesting that it could fit into the NEP cleavage pocket (Figure 2E).

**Assess the role of heparanoids in regulating bFGF susceptibility to proteolytic cleavage.**

Basic FGF is primarily stored in the extracellular matrix and basement membrane associated with heparan sulfate proteoglycan (HSPG). Activity of bFGF is controlled in part by a low-affinity but high-capacity interaction with HSPG. Free bFGF may be proteolytically degraded, as suggested by in vitro reactivity of the C-terminal portion of bFGF to trypsin and chymotrypsin (Kajio et al., 1992; Sommer and Rifkin, 1989). We hypothesized that HSPG binding could function to protect bFGF from degradation by NEP since leucine 135 and glycine 136 of the bFGF protein lie within a basic region where heparin-derived tetra- and hexasaccharides have been reported to complex with bFGF (Faham et al., 1996). Incubation of rNEP and bFGF plus heparin (12 units/ml) showed that heparin completely inhibited the ability of NEP to cleave bFGF (Figure 3, lane 2 compared to lane 3). Together, these data suggest that bFGF is a substrate for NEP, and that HSPG protects bFGF from NEP cleavage.

**Characterize the actions of bFGF cleavage products on FGF receptor signal transduction pathways, and on the biology of human vascular endothelial cells.** To establish that NEP antiangiogenic activity is a direct consequence of bFGF cleavage, we examined whether cleavage of bFGF abrogated its ability to signal through the fibroblast growth factor receptor (FGF-R). Upon engaging bFGF, FGF-R undergoes dimerization, autophosphorylation and then signals by way of the mitogen-activated protein kinase pathway resulting in extracellular signal-related kinase (ERK) phosphorylation (Nugent and Iozzo, 2000). Therefore, we first assessed rNEP inhibition of bFGF signaling by blotting HUVEC lysates for phosphorylated ERK (P-ERK) and total ERK (T-ERK) prepared following treatment with either bFGF (100 ng/mL) alone, or bFGF preincubated with rNEP. As shown in Figure 4A, rNEP treatment decreased bFGF-induced ERK phosphorylation by ~50%. As a confirmatory approach, we next examined the effect of NEP inhibition on ERK phosphorylation following treatment with a tenfold lower concentration of bFGF (10 ng/mL). At this concentration neither bFGF nor inhibition of endogenous NEP with CGS24592 resulted in appreciable stimulation of ERK phosphorylation (Figure 4B), however CGS24592 treatment led to a bFGF-stimulated increase in ERK phosphorylation.

Next we sought to identify the mechanism, either loss of receptor binding or receptor antagonism, by which bFGF cleavage results in its inactivation. This was done by simultaneously examining cell surface binding of bFGF cleavage products and their ability to induce ERK phosphorylation. On incubation of GST-bFGF fusion proteins with intact HUVEC and tHBMEC, bFGF cleavage products but not full length bFGF constructs failed to signal through FGF-R (Figure 4C) and coincidentally failed to bind to cultured vascular endothelial cells (Figure 4D), implying that NEP cleavage reduces bFGF signaling by rendering bFGF incapable of binding cell surface receptor.

*Aim 2: To delineate the anti-angiogenic action of NEP on vascular endothelial cells.*

**Endogenous NEP expressed on human vascular endothelial cells negatively regulates bFGF-induced angiogenesis.** Previous studies indicate that NEP is expressed by human

vascular endothelial cells (Graf et al., 1995; Llorens-Cortes et al., 1992). Analyses of HUVEC and SV-40 transduced human bone marrow endothelial cells (tHBMEC, (Schweitzer et al., 1997) revealed NEP enzyme specific activities of 36 pmol/ $\mu$ g/min and 197 pmol/ $\mu$ g/min, respectively (data not shown). To assess the functional effect of endogenous NEP on bFGF-induced angiogenesis in vitro, we used a capillary tube formation assay to measure the effects of bFGF with and without the NEP inhibitor CGS24592 in tHBMEC cells plated on matrigel coated plates. As shown in Figure 5A, cells grown in the presence of bFGF and CGS24592 demonstrated significantly more capillary tube formation compared to cells grown in bFGF alone, CGS24592 alone or the untreated negative control ( $P < 0.05$ ). We next assessed endothelial cell growth in tHBMEC treated with 600 pg/mL bFGF as a function of increasing concentrations of CGS24592. Transduced HBMEC incorporated more MTT in the presence of CGS24592, suggesting that inhibition of endogenous NEP activity results in increased bFGF-induced cell growth ( $P < 0.03$ , Figure 5B). Similar results were obtained for HUVEC cells (data not shown). These data show that NEP expressed on vascular endothelial cells regulates bFGF induced angiogenesis.

**Basic FGF cleavage products do not induce angiogenesis.** To confirm that NEP cleavage inactivates bFGF function, we produced and purified glutathione-S-transferase (GST) proteins fused to full-length bFGF, bFGF cleavage products corresponding to amino acids 1-135 and 136-155, and as negative control, GST alone, and tested their ability to promote capillary tube formation in primary HUVEC cultures in the presence of the NEP inhibitors phosphoramidon (PPA, Figure 6A) and CGS24592 (data not shown). Recombinant and full length GST-bFGF protein promoted similar amounts of tube formation. However, neither the 1-135 nor the 136-155 bFGF cleavage products demonstrated any biologic activity in this assay.

Mutagenesis of the putative NEP cleavage site residues to alanine resulted in stepwise decrease in NEP cleavage, with the double mutant L135A/G136A demonstrating a near complete loss of cleavage by NEP (Figure 6B). When tested in a matrigel tube formation, the NEP-resistant bFGF was capable of inducing tube formation on matrigel comparable to that seen with a 10-fold higher concentration of bFGF or with CGS24592 treatment (Figure 6C). Taken together, these data suggest that endogenously expressed NEP on vascular endothelial cells regulates bFGF-induced angiogenesis.

**Assess the role of heparanoids in regulating bFGF susceptibility to proteolytic cleavage.** Heparin sulfate can inhibit proteolysis of bFGF by NEP (Figure 3). Experiments to test the ability of bFGF cleavage products for heparin binding are ongoing, as are those to assess the role of proteoglycan sulfation in modulating the sensitivity of bFGF to NEP cleavage

**Assess the pro-angiogenic activity of bFGF in NEP null mice compared to wild-type mice.** Experiments utilizing the murine corneal pocket assay are currently ongoing. A cohort of mice subjected to DMBA-induced skin carcinogenesis failed to demonstrate a increased rate of conversion. Given that there was a 8% incidence of tumor formation the wildtype and 14% incidence in the NEP null mice (data not shown), considerably lower than that observed in other backgrounds (50-80%), we conclude that the assay will not be adequately powered statistically to detect a difference in our strain. We therefore will establish a syngeneic tumor model system in the upcoming year. As a complementary strategy, we are in the process of isolating endothelial cells from murine lung, hear and aorta using immunoselection with magnetic beads (Dynal). We



will assess these cell lines for their responsiveness to stimulation with bFGF, assessing them with respect to cell growth, capillary tube formation, and ERK signaling.

**Decipher the hypoxic-dependent of NEP expression in human vascular endothelial cells.**

Experiments to characterize the negative regulation of NEP activity by hypoxia are ongoing and are expected to be completed within the upcoming year of grant support.

**c. Significance.** Prostate cancer is the most common primary cancer among men and the second leading cause of cancer deaths in males in the United States. Our previous studies have shown that NEP loss is involved in the development and progression of both early and advanced hormone refractory PC. Moreover, we now show that complete loss of NEP expression in primary PC is associated with a shorter time to PSA recurrence, suggesting that in addition to NEP loss contributing to neuropeptide-mediated PC progression, it permits more effective malignant angiogenesis. Understanding the molecular events involved in the development and progression of PC is critical to developing more effective therapies. These studies, leading to a better understanding of the involvement of NEP in the development and progression of PC, may ultimately provide support for novel antioangiogenic approaches for the treatment of advanced PC. In contrast, conditions where angiogenesis is pathologically impaired such as ischemic vascular disease, may benefit from so-called “super bFGFs” which resist proteolysis by NEP and therefore may be more potent angiogenic agents.

**d. Plans.** Our continuing objective is to elucidate and to understand the involvement of NEP with regard to its anti-angiogenic action. In the second year of grant support, we will continue our studies aimed at understanding the mechanisms of NEP induced inhibition of angiogenesis. Our studies have provided us thus far with a detailed understanding of the role NEP plays in inactivating bFGF. We will further our analysis by determining the role of the NEP cytoplasmic tail plays in NEP action and signaling in vascular endothelial cells. We will pursue studies to better understand the inhibitory role that heparin sulfate proteoglycans play in modulating the bFGF cleaving activity of NEP. In addition, we will use the NEP null murine model to better understand the anti-angiogenic role of NEP *in vivo*.

**e. Key Research Accomplishments**

Goodman OB, Febbraio M, Simantov R, Shen R, Silverstein R, Nanus, DM. Neutral endopeptidase 24.11 (NEP) functions as a negative regulator of angiogenesis *in vivo* by proteolytic cleavage of basic fibroblast growth factor (2005), manuscript in preparation.

**f. Reportable Outcome:**

Dr. Goodman was awarded an American Society of Clinical Oncology Young Investigator Award with support from 7/01/05-6/30/06

### **Conclusions**

- NEP inactivates bFGF by proteolytic cleavage near its C-terminus, a novel antiangiogenic mechanism
- bFGF cleavage products are functionally inactive on vascular endothelial cells due their inability to bind to engage surface receptor and signal through mitogen activated protein kinase.
- NEP inhibition augments bFGF-induced vascular endothelial cell growth and capillary tube formation.
- Hypoxia negatively regulate NEP activity in endothelial and PC cells

## References

- Abraham, J.A., Whang, J.L., Tumolo, A., Mergia, A. and Fiddes, J.C. (1986) Human basic fibroblast growth factor: nucleotide sequence, genomic organization, and expression in mammalian cells. *Cold Spring Harb Symp Quant Biol*, 51 Pt 1, 657-668.
- Bagnato, A. and Spinella, F. (2003) Emerging role of endothelin-1 in tumor angiogenesis. *Trends Endocrinol Metab*, 14, 44-50.
- Dai, J., Shen, R., Sumitomo, M., Goldberg, J.S., Geng, Y., Navarro, D., Xu, S., Koutcher, J.A., Garzotto, M., Powell, C.T. and Nanus, D.M. (2001) Tumor-suppressive effects of neutral endopeptidase in androgen-independent prostate cancer cells. *Clin Cancer Res*, 7, 1370-1377.
- Faham, S., Hileman, R.E., Fromm, J.R., Linhardt, R.J. and Rees, D.C. (1996) Heparin structure and interactions with basic fibroblast growth factor. *Science*, 271, 1116-1120.
- Graf, K., Koehne, P., Grafe, M., Zhang, M., Auch-Schwelk, W. and Fleck, E. (1995) Regulation and differential expression of neutral endopeptidase 24.11 in human endothelial cells. *Hypertension*, 26, 230-235.
- Howell, S., Nalbantoglu, J. and Crine, P. (1995) Neutral endopeptidase can hydrolyze beta-amyloid(1-40) but shows no effect on beta-amyloid precursor protein metabolism. *Peptides*, 16, 647-652.
- Iwase, A., Shen, R., Navarro, D. and Nanus, D.M. (2004) Direct binding of neutral endopeptidase 24.11 to ezrin/radixin/moesin (ERM) proteins competes with the interaction of CD44 with ERM proteins. *J Biol Chem*, 279, 11898-11905.
- Kajio, T., Kawahara, K. and Kato, K. (1992) Stabilization of basic fibroblast growth factor with dextran sulfate. *FEBS Lett*, 306, 243-246.
- Kastrup, J.S., Eriksson, E.S. (1997) X-ray structure of the 154-amino-acid form of recombinant human basic fibroblast growth factor. Comparison with the truncated 146-amino-acid form. *Acta Crystallogr D Biol Crystallogr*, 53, 160-168.
- Kenny, J. (1993) Endopeptidase-24.11: putative substrates and possible roles. *Biochem Soc Trans*, 21 (Pt 3), 663-668.
- Llorens-Cortes, C., Huang, H., Vicart, P., Gasc, J.M., Paulin, D. and Corvol, P. (1992) Identification and characterization of neutral endopeptidase in endothelial cells from venous or arterial origins. *J Biol Chem*, 267, 14012-14018.
- Maniara, W.M., Cipriano, A. and Powell, M.L. (1998) Quantitative analytical methods for the determination of a new hypertension drug, CGS 25462, and its metabolites (CGS 25659 and CGS 24592) in human plasma by high-performance liquid chromatography. *J Chromatogr B Biomed Sci Appl*, 706, 287-294.
- Nanus, D.M. (2003) Of peptides and peptidases: the role of cell surface peptidases in cancer. *Clin Cancer Res*, 9, 6307-6309.
- Nugent, M.A. and Iozzo, R.V. (2000) Fibroblast growth factor-2. *Int J Biochem Cell Biol*, 32, 115-120.
- Osman, I., Yee, H., Taneja, S.S., Levinson, B., Zeleniuch-Jacquotte, A., Chang, C., Nobert, C. and Nanus, D.M. (2004) Neutral endopeptidase protein expression and prognosis in localized prostate cancer. *Clin Cancer Res*, 10, 4096-4100.
- Papandreou, C.N., Usmani, B., Geng, Y., Bogenrieder, T., Freeman, R., Wilk, S., Finstad, C.L., Reuter, V.E., Powell, C.T., Scheinberg, D., Magill, C., Scher, H.I., Albino, A.P. and Nanus, D.M. (1998) Neutral endopeptidase 24.11 loss in metastatic human prostate cancer contributes to androgen-independent progression. *Nat Med*, 4, 50-57.

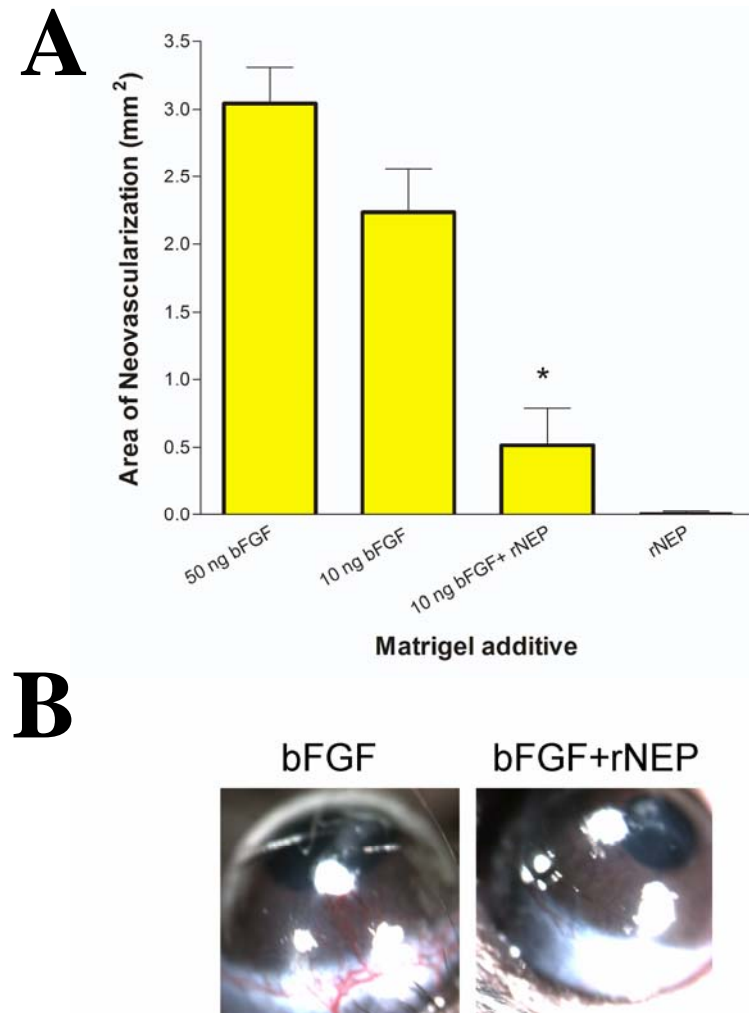
- Schweitzer, K.M., Vicart, P., Delouis, C., Paulin, D., Drager, A.M., Langenhuijsen, M.M. and Weksler, B.B. (1997) Characterization of a newly established human bone marrow endothelial cell line: distinct adhesive properties for hematopoietic progenitors compared with human umbilical vein endothelial cells. *Lab Invest*, 76, 25-36.
- Sommer, A. and Rifkin, D.B. (1989) Interaction of heparin with human basic fibroblast growth factor: protection of the angiogenic protein from proteolytic degradation by a glycosaminoglycan. *J Cell Physiol*, 138, 215-220.
- Sumitomo, M., Iwase, A., Zheng, R., Navarro, D., Kaminetzky, D., Shen, R., Georgescu, M.M. and Nanus, D.M. (2004) Synergy in tumor suppression by direct interaction of neutral endopeptidase with PTEN. *Cancer Cell*, 5, 67-78.
- Sumitomo, M., Milowsky, M.I., Shen, R., Navarro, D., Dai, J., Asano, T., Hayakawa, M. and Nanus, D.M. (2001) Neutral endopeptidase inhibits neuropeptide-mediated transactivation of the insulin-like growth factor receptor-Akt cell survival pathway. *Cancer Res*, 61, 3294-3298.
- Sumitomo, M., Shen, R., Walburg, M., Dai, J., Geng, Y., Navarro, D., Boileau, G., Papandreou, C.N., Giancotti, F.G., Knudsen, B. and Nanus, D.M. (2000) Neutral endopeptidase inhibits prostate cancer cell migration by blocking focal adhesion kinase signaling. *J Clin Invest*, 106, 1399-1407.

## **Appendices**

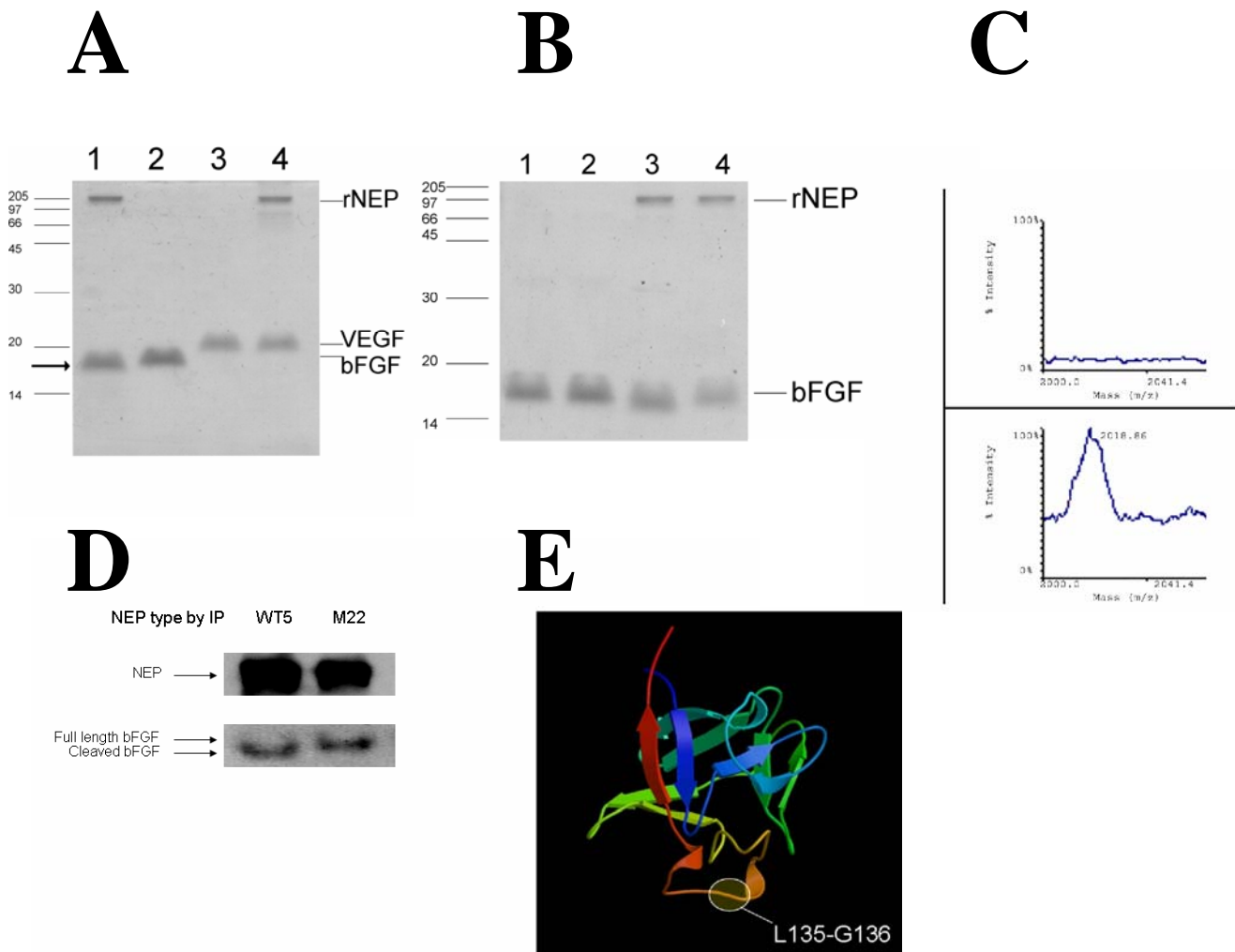
### **Abbreviations:**

Ab: antibody  
AR: androgen receptor  
bFGF: basic fibroblast growth factor  
ECE: endothelin converting enzyme  
ET-1: Endothelin-1  
ERM: ezrin/radixin/moesin  
FAK: focal adhesion kinase  
FGFR2: FGF receptor-2  
HSPG: heparin sulfate proteoglycans  
IGF-1R: insulin-like growth factor-1 receptor  
mAb: monoclonal antibody  
NEP: neutral endopeptidase  
PC: prostate cancer  
PI3-K: phosphatidylinositol 3-kinase  
rNEP: recombinant NEP  
tHBMEC:SV-40 transduced human bone marrow microvascular endothelial cells

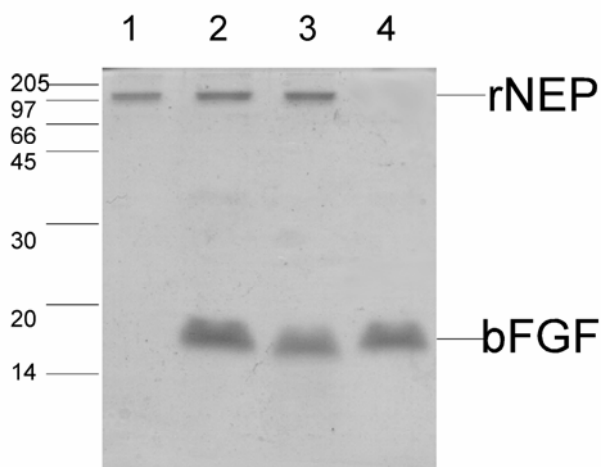
**Figures:**



**Figure 1. Recombinant NEP inhibits bFGF-induced angiogenesis.** A) Pellets containing 50 ng bFGF (positive control), 10 ng bFGF, 10 ng bFGF + 50 ng rNEP or 50 ng rNEP alone (negative control) were implanted in the cornea of C57/B6 mice and new vessel formation at 5 days measured by slit lamp ophthalmoscopy. Statistical analysis of six eyes (3 mice) in two independent experiments (a total of 6 mice for each group) was performed (\* denotes  $P < 0.01$  compared to 10 ng bFGF, 2-tailed unpaired t-test). B) Photograph of representative corneas: 10 ng bFGF alone on left, 10 ng bFGF + 50 ng rNEP on right.

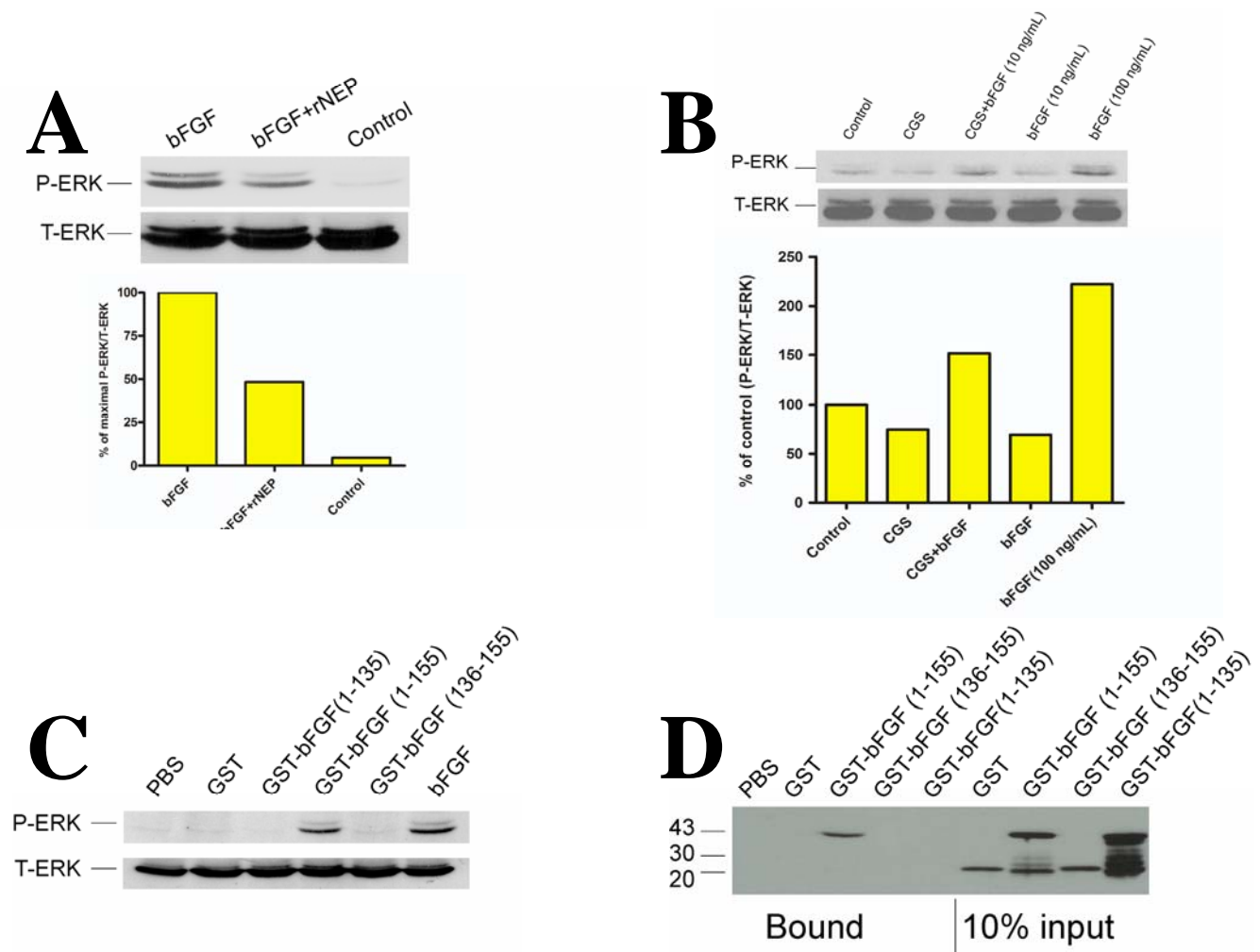


**Figure 2. NEP Enzymatic Activity Cleaves bFGF Protein.** A) Basic FGF or VEGF was incubated with or without rNEP for 1 hour at 25 °C in 100mM Tris-HCl, pH7.6 buffer, and the samples separated by 14% SDS-PAGE. Lane 1: 12.5  $\mu$ M bFGF + 1  $\mu$ M rNEP; Lane 2: 12.5  $\mu$ M bFGF alone; Lane 3: 12.5  $\mu$ M VEGF alone; Lane 4: 12.5  $\mu$ M VEGF + 1  $\mu$ M rNEP. Note faster migration of bFGF protein treated with rNEP (arrow). B) 12.5  $\mu$ M of bFGF was incubated with or without rNEP and the NEP inhibitor CGS24592 for 1 hour at 25 °C, and the samples separated by 14% SDS-PAGE. Lane 1: DMSO vehicle (control); Lane 2: 3 $\mu$ M CGS24592; Lane 3: 1  $\mu$ M rNEP; Lane 4: 1 $\mu$ M rNEP + 3 $\mu$ M CGS24592. Note that the addition of CGS24592 blocks the faster migration of bFGF protein treated with rNEP (Lane 4). C) Recombinant NEP was incubated with recombinant bFGF with (upper panel) or without (lower panel) CGS24592 and analyzed by MALDI-TOFMS. Note the presence of a 2019 Da band, which corresponds to amino acids 136-155 of the bFGF protein in the absence of CGS24592. D) Lysates from WT-5 and M22 cells cultured in tetracycline-free media for 48 hours containing 500  $\mu$ g total protein were subjected to immunoprecipitation with J5 antibody and incubated with 100 ng of bFGF for 4 hours at 37°C. Samples were analyzed with SDS-PAGE and Western blotting for both bFGF and NEP. Note the increased electrophoretic mobility seen with wildtype NEP (WT5) but not enzymatically deficient NEP (M22). E) Crystal structure of the 155-amino-acid form of recombinant bFGF (PDB ID: 1BFF from Kastrup, 1997). The putative NEP cleavage site is highlighted.

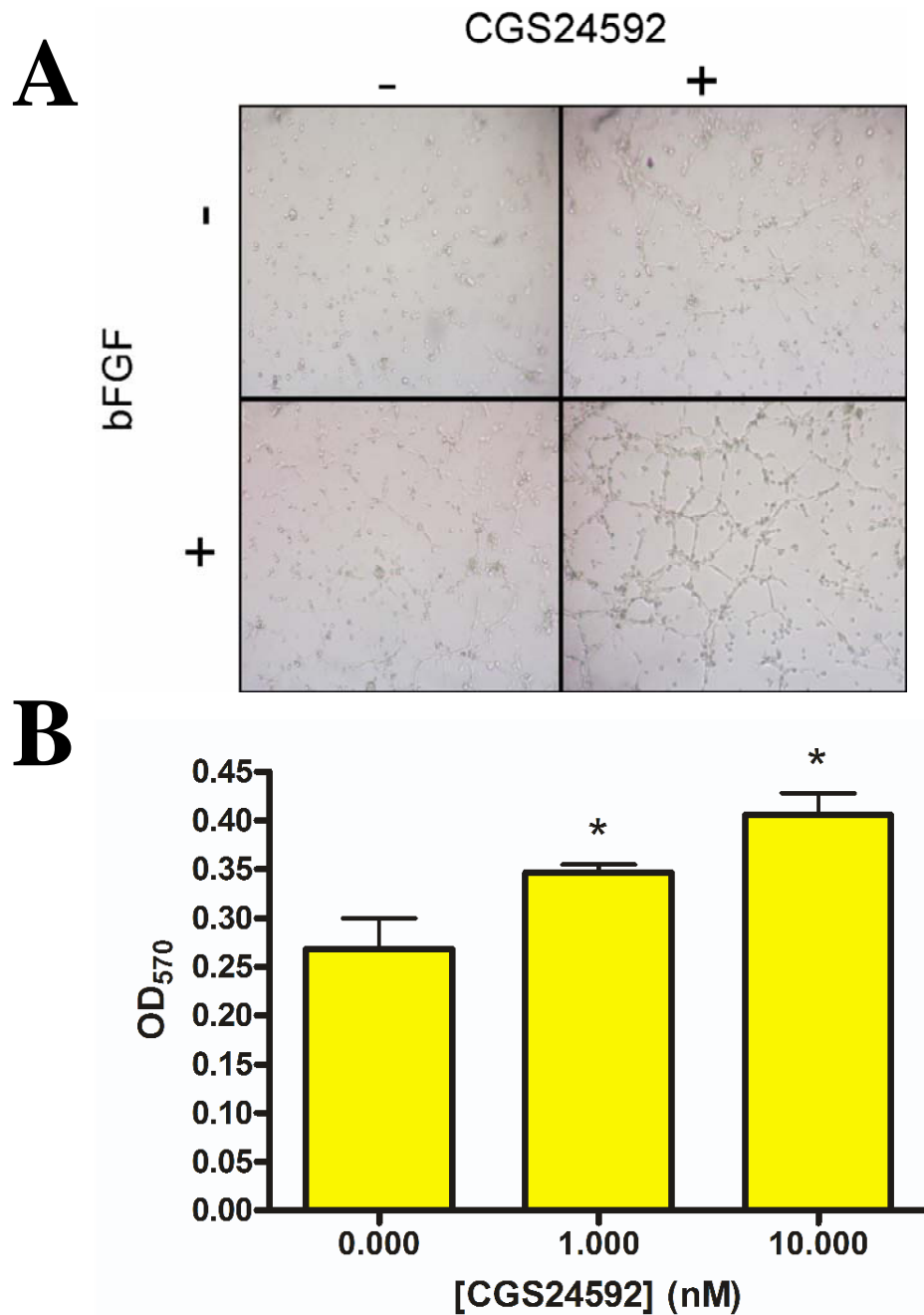


**Figure 3. Heparin sulfate inhibits NEP cleavage of bFGF protein.** Basic fibroblast growth factor, rNEP and heparin were incubated for 1 hour at 25 °C in 100 mM Tris-HCl, pH7.6 and subjected to 14% SDS-PAGE analysis with Coomassie blue staining. Lane 1: 1  $\mu$ M rNEP alone; Lane 2: 12.5  $\mu$ M bFGF + 1  $\mu$ M rNEP +12 U/mL heparin; Lane 3: 12.5  $\mu$ M bFGF + 1  $\mu$ M rNEP; Lane 4: 12.5  $\mu$ M bFGF alone. Note heparin inhibits the faster migration of bFGF protein treated with rNEP (Lane 2). Lanes 1 and 4 are controls.

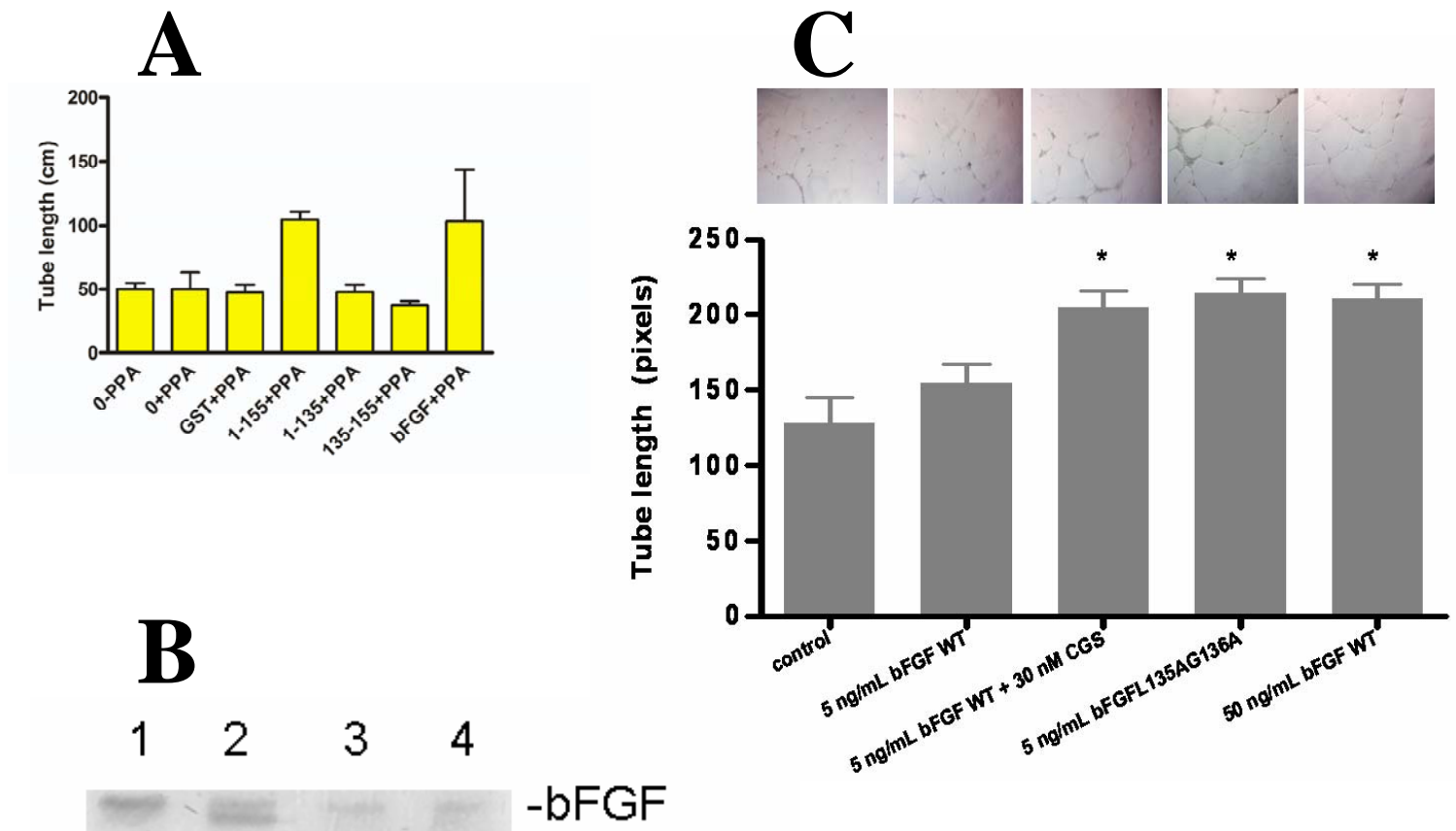




**Figure 4. NEP inhibition of bFGF signaling through MAP-kinase.** A) NEP attenuation of bFGF signaling through ERK. bFGF (8  $\mu$ g/mL) was incubated in the presence or absence of rNEP (60  $\mu$ g/mL) for 1 hour at 25°C in 100 mM Tris-HCl pH 7.6 and then added to subconfluent HUVEC monolayers for a bFGF concentration of 100 ng/mL, incubated for 20 minutes, harvested with RIPA lysis buffer and 50  $\mu$ g of protein subjected to Western blotting analysis using anti-total ERK antibody (T-ERK) or anti-phosphorylated ERK antibody (P-ERK). As negative control, buffer alone was used. Results are representative of three independent experiments. The inset shows densitometric analysis of the included experiment. B) Potentiation of bFGF signaling through ERK. As in 4A above, bFGF and rNEP were incubated and then added to subconfluent HUVEC monolayers which were pretreated with 10 nM CGS24592 or vehicle for two hours, for a final bFGF concentration of 10 ng/mL, incubated for 20 minutes, harvested with RIPA lysis buffer and 50  $\mu$ g of protein subjected to Western blotting using T-ERK or P-ERK antibodies. The inset shows densitometric analysis of the included experiment. C) Characterization of bFGF cleavage products with regard to cell signaling. Subconfluent HUVEC monolayers were treated with 5 nM of recombinant proteins as indicated for 20 minutes and washed 3 times with 2 mL PBS. RIPA cell lysates were analyzed for total and phosphorylated ERK as in 4A. Recombinant bFGF was used as positive control. D) Testing of bFGF cleavage products for binding to intact cells. Lysates from C above were analyzed by SDS-PAGE, blotted with anti-GST antibody and compared to 10% input standards.



**Figure 5. Endogenous NEP inhibition stimulates bFGF-induced angiogenesis.** A) Transduced human bone marrow microvascular endothelial cells were assayed using a matrigel capillary tube formation assay. Cells were plated on growth factor reduced matrigel in the presence (+) or absence(-) of CGS24592 for 2 hours followed by the addition of bFGF for 4 hours as indicated. Representative photographs were then taken. B) Transduced human bone marrow microvascular endothelial cells were incubated with 600 pg/mL bFGF in DMEM supplemented with 1% FCS and increasing concentrations of CGS24592. After 48 hours, media was exchanged with that containing MTT at a concentration of 0.5 mg/ml and incubation continued for four more hours. Incorporated MTT was liberated with DMSO, and expressed as OD<sub>570nm</sub> (\* denotes P<0.03 relative to no CGS24592). Results are representative of two independent experiments with similar results performed in triplicate.



**Figure 6. NEP cleavage of bFGF is necessary and sufficient to abrogate its proangiogenic function.** A) 15,000 HUVEC cells were plated on growth factor reduced matrigel in the presence or absence of 30  $\mu$ M phosphoramidon, and adding the indicated commercially available bFGF or GST-bFGF fusion protein to a final concentration of 50 nM. Tube formation was quantified after 18 hours by measuring total length of capillary tube formed per 500X field using ImageJ software. Results are representative of 3 independent experiments with similar results performed in triplicate (\* denotes  $P < 0.05$  relative to GST control). B) Wildtype bFGF (Lanes 1-2) and the bFGF mutant L135A/G136A (Lanes 2-4) were incubated with 1  $\mu$ g NEP (lanes 2 and 4) or vehicle (lanes 1 and 3) at 25 degrees for 6 hours and then subjected to SDS-PAGE analysis. As seen, wildtype but not mutant form of bFGF undergoes a electrophoretic shift. C) 15,000 HUVEC cells were plated on growth factor reduced matrigel in the presence or absence of 30 nM CGS 24592, and the indicated bFGF fusion protein (5-50 ng/mL). Tube formation was quantified after 18 hours by measuring total length of capillary tube formed per 500X field in five different fields using ImageJ software. Results are representative of 3 independent experiments with similar results performed in duplicate (\* $P < 0.01$  relative to control and wildtype bFGF).